



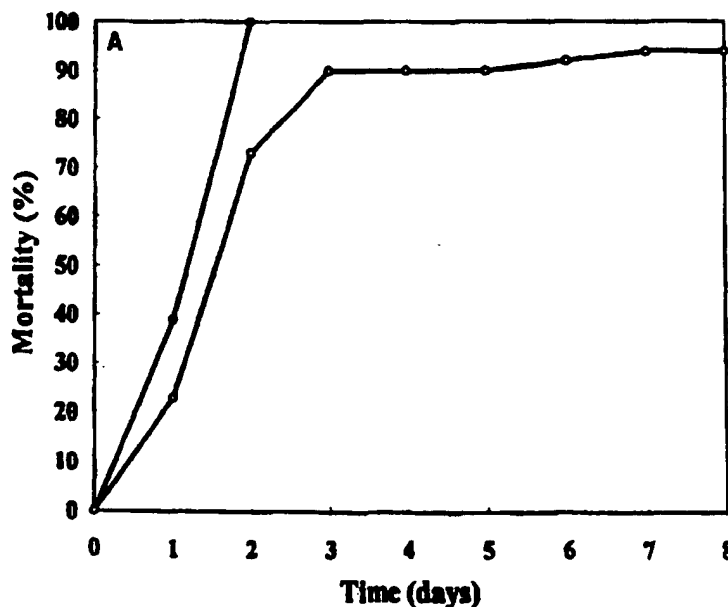
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 7/01, 15/33, 15/32, 15/62, A61K 39/12	A1	(11) International Publication Number: WO 99/51721 (43) International Publication Date: 14 October 1999 (14.10.99)
(21) International Application Number: PCT/KR98/00338 (22) International Filing Date: 28 October 1998 (28.10.98) (30) Priority Data: 1998/11914 3 April 1998 (03.04.98) KR (71)(72) Applicants and Inventors: KANG, Seok, Kwon [KR/KR]; 104-801, Hyundai APT, Maetan-dong, Paldal-ku, Suwon-city, Kyunggi-do 442-370 (KR). JE, Yeon, Ho [KR/KR]; 45-71, Seodoon-dong, Kwonsun-ku, Suwon-city, Kyunggi-do 441-100 (KR). JIN, Byung, Rae [KR/KR]; 205, Kyokwan APT, Seodoon-dong, Kwonsun-ku, Suwon-city, Kyunggi-do 441-100 (KR). (72) Inventors: PARK, Hyun, Woo; 18-81, Shibum APT, Yoido-dong, Youngdungpo-ku, Seoul 150-010 (KR). ROH, Jong, Yul; 28/4, 1000-3, Sadang-4-dong, Tongjak-ku, Seoul 156-094 (KR). CHANG, Jin, Hee; 21-1206, Hanyang APT, Songpa-dong, Songpa-ku, Seoul 138-170 (KR). (74) Agent: LEE, Duck, Rog; Yorksam Building, 2nd floor, 700-19, Yorksam-dong, Kangnam-ku, Seoul 135-080 (KR).		(81) Designated States: AU, BR, CA, CN, JP, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.</i>

(54) Title: NOVEL RECOMBINANT BACULOVIRUS, CONSTRUCTION METHOD THEREOF AND INSECT PESTICIDAL COMPOSITION CONTAINING THE SAME

(57) Abstract

A recombinant baculovirus, which produces a recombinant polyhedra made up of a baculovirus polyhedrin (PH), a *Bacillus thuringiensis* crystal protein (CP) and jellyfish *Aequorea victoria* green fluorescent protein (GFP), is constructed by introducing a transfer vector carrying a fusion gene encoding a fusion protein in which the PH, the CP and the GFP are directly linked from N-terminal to C-terminal, in sequence, and a wild-type baculovirus into an insect cell, simultaneously, and culturing the cell. This baculovirus transfer vector pColorBtrus is constructed by synthesizing the GFP-coding DNA fragment from plasmid pGFP, the PH gene from wild-type *Autographa californica* Nucleopolyhedrovirus, and a CryIAc gene from plasmid pPN6.6 carrying a *Bacillus thuringiensis* CryIAc gene by polymerase chain reactions, and inserting first the GFP-coding DNA fragment and the PH gene in baculovirus expression vector pAcUW31 in such a way that the 5'-end of the GFP-coding DNA fragment is linked to the 3'-end of the PH gene and then, the CryIAc gene between the PH gene and the GFP-coding DNA fragment. The recombinant virus can kill insect pests with high pathogenicity within a short time, and is equipped with a monitoring device for infected insects, so that it can be prevented from being overused.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CJ	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**NOVEL RECOMBINANT BACULOVIRUS, CONSTRUCTION METHOD
THEREOF AND INSECT PESTICIDAL COMPOSITION CONTAINING THE
SAME**

5 **TECHNICAL FIELD**

The present invention relates to a novel recombinant baculovirus which exhibits quick and potent insecticidal activity, a construction method thereof and an insect
10 pesticial composition comprising the same.

BACKGROUND ART

Baculoviruses are pathogenic to insects, so that they
15 are proliferated to kill the hosts. In addition, baculoviruses are nonpathogenic to the vertebrate and maintain their activity for a long time in fields. Owing to these advantages, as many as 20 viruses are developed as commercially available insecticides. However,
20 baculoviruses have a limited host range and the insecticidal effects are relatively slow. These disadvantages have limited their use as control agents of insect pests.

Recently, active research has been and continues to be directed to the improvement of baculoviruses in usability as
25 specific pest control agents. In this regard, genetic

engineering technology provides a useful means therefor. In fact, a number of attempts have been made to produce the baculoviruses which are improved in killing speed, by introducing various foreign genes into the baculoviruses.

5 The foreign genes with this purpose include those coding for *Buthus eupeus* insect toxin-1 (Carbonell et al., *Gene*, 73, 409-418 (1988)), *Manduca sexta* diuretic hormone (Maeda, *Biochem. Biophys. Res. Commun.*, 165, 1177-1183 (1989)), *Heliothis virescens* juvenile hormone esterase (Hammock et
10 al., *Nature*, 344, 458-461 (1990); Bonning et al., *Insect Biochem. Mol. Biol.*, 22, 453-458 (1992)), *Pyemotes tritici* TxP-I toxin (Tomalski and Miller, *nature*, 352, 82-85 (1991)), *Androctonus australis* Aa1T toxin (Stewart et al., *Nature*, 352, 85-88) and insect-specific spider toxin (Hughes
15 et al., *J. Invertebr. Pathol.*, 69, 112-118 (1997)). Of them, only the gene encoding scorpion toxin allowed the recombinant baculoviruses to show an increased pathogenicity. The killing speed, however, remains slow even in the baculoviruses carrying this gene.

20 The delta-endotoxin of *Bacillus thuringiensis* (a gram positive bacteria, hereinafter referred to as "Bt"), an insect-specific toxin which accumulates in large amounts during the sporulation of Bt, has been demonstrated as an effective means of controlling pest populations by virtue of
25 its potent insecticidal activity and nontoxicity to humans

and poultry (Feitelson et al., *Bio/Technology*, 10, 271-275 (1992)). As for the insecticidal mechanism of the endotoxin, it starts by enzyme cleavage. When taken in by a susceptible insect, the endotoxin is solubilized in the alkaline environment of the gut and cleaved to a smaller active protein by the action of proteases present in the gut juices. The activated protein binds to the epithelial cells of the gut, leading to disruption of the gut. While showing paralytic symptoms owing to the change in the pH of the digestive juice and humor, the susceptible insects cease to eat food and are finally put to death in 24-48 hours. For instance, the lepidoptera specific crystal protein (Cry 1), a kind of Bt endotoxin, is 130-140 kDa in total, but its toxicity is greatly enhanced if the protein is cleaved by proteases to a highly active fragment of about 60-70 kDa which corresponds to the N-terminal half of the protein (Aronson, et al., *Microbiol. Rev.*, 50, 1-24 (1986)).

There were noticeable studies on the improvement of viral insecticidal activity by expressing the Bt endotoxin protein in baculoviruses (Bonning and Hammock, *Annu. Rev. Entomol.*, 41, 191-210 (1996)). However, there was observed no enhancement in pathogenicity of the recombinant baculoviruses although they produce a large amount of the Bt endotoxin proteins in the blood lymphs of the insects. This results from no consideration for the insecticidal mechanism

of Bt endotoxin which is fulfilled in the presence of the proteases and the epithelial cells in the gut. That is, the single Bt endotoxin protein which is expressed under the control of the promotor Ppol in the blood lymphs, is not
5 activated as in the gut.

DISCLOSURE OF THE INVENTION

Bearing the activation of the baculovirus polyhedrin by
10 protease in the alkaline environment of the insect gut in mind, the present inventors repeated thorough and intensive research with the aim of developing a novel baculovirus which can kill insect pests with high pathogenicity within a short time and finally, resulted in finding that fusion of
15 *Bacillus thuringiensis* crystal protein with the baculovirus polyhedrin allows a significant improvement in pathogenicity and killing time.

Therefore, it is an object of the present invention to overcome the above problems encountered in prior arts and to
20 provide a novel recombinant baculovirus which can exhibit pathogenicity within a short time.

It is another object of the present invention to provide a novel recombinant baculovirus which is equipped with a monitoring device for infected insects.

25 It is a further object of the present invention to

provide a construction method of the recombinant baculovirus.

It is still a further object of the present invention to provide a method of constructing a transfer vector for the recombinant baculovirus.

It is still another object of the present invention to provide an insect pesticidal composition which is of potent insecticidal activity and quickness and preventive of overuse.

10 It is yet another object of the present invention to provide a simple purification method of proteins.

According to an aspect of the present invention, there is provided a recombinant baculovirus, which produces a recombinant polyhedra made up of a baculovirus polyhedrin (PH), a *Bacillus thuringiensis* crystal protein (CP) and jellyfish *Aequorea victoria* green fluorescent protein (GFP).

According to another aspect of the present invention, there is provided a construction method of baculovirus transfer vector pColorBtrus, comprising the steps of
20 synthesizing a green fluorescent protein (GFP)-coding DNA fragment from plasmid pGFP by a polymerase chain reaction, synthesizing a polyhedrin (PH) gene from wild-type *Autographa californica* Nucleopolyhedrovirus by a polymerase chain reaction, inserting the green fluorescent protein-
25 coding DNA fragment and the polyhedrin gene in baculovirus

expression vector pAcUW31 in such a way that the 5'-end of the green fluorescent protein-coding DNA fragment is linked to the 3'-end of the polyhedrin gene, to yield baculovirus transfer vector pColorPol, synthesizing a Cry1Ac gene from 5 plasmid pPN6.6 carrying a *Bacillus thuringiensis* Cry1Ac gene by a polymerase chain reaction, and inserting the Cry1Ac gene between the polyhedrin gene and the green fluorescent protein-coding DNA fragment.

According to a further object of the present invention, 10 there is provided a method of constructing the recombinant baculovirus, comprising the steps of introducing a transfer vector carrying a fusion gene encoding a fusion protein in which a baculovirus polyhedrin, a *Bacillus thuringiensis* crystal protein and a jellyfish *Aequorea victoria* green 15 fluorescent protein are directly linked from N-terminal to C-terminal, in sequence, and a wild-type baculovirus into an insect cell, simultaneously, and culturing the cell.

According to a further object of the present invention to provide an insecticidal composition which comprises the 20 recombinant virus.

According to still another object of the present invention, there is provided a purification method of proteins using the transfer vector system.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1a, 1b and 1c are baculovirus transfer vector maps of pAcG, pColorPol and pColorBtrus, respectively.

Figs. 2a and 2b are respectively light and fluorescent microphotographs, showing Sf9 cells infected with control 5 (wild-type) AcNPV.

Figs. 2c and 2d are respectively light and fluorescent microphotographs, showing sf9 cells infected with recombinant baculovirus AcG.

Figs. 2e and 2f are respectively light and fluorescent 10 microphotographs, showing sf9 cells infected with recombinant baculovirus ColorPol.

Figs 2g and 2h are respectively light and fluorescent microphotographs, showing sf9 cells infected with recombinant baculovirus ColorBtrus.

15 Figs. 3a, 3b and 3c show the western blots obtained from the recombinant polyhedra by use of anti-GFP antibody, anti-PH antibody and anti-Cry1Ac antibody, respectively.

Figs. 4a and 4b are transmission electron microphotographs showing the immunogold-labeled recombinant 20 polyhedra of wild-type AcNPV and the recombinant ColorBtrus polyhedra, respectively.

Fig. 5 shows Western blots obtained from the recombinant polyhedra by use of an anti-Cry1Ac antibody.

Fig. 6 is a graph showing the accumulated insecticidal 25 activity of recombinant baculovirus ColorBtrus against *P.*

Xylostella larvae.

Fig. 7a is a photograph showing *P. xylostella* larvae which were infected with the recombinant polyhedra of ColorBtrus three days earlier, and Fig. 7b is a fluorescent microphotograph showing the fat body from the dead larvae which was infected with ColorBtrus six days earlier.

Figs. 8a and 8b are microphotographs showing a reduction in feeding damage by ColorBtrus-infected *P. xylostella* larvae on Chinese cabbage leaves which the polyhedra of ColorBtrus and wild-type AcNPV were treated, respectively.

BEST MODES FOR CARRYING OUT THE INVENTION

Throughout the specification, the term baculoviruses mean the virions embedded in polyhedra. The polyhedra is an assembly of polyhedron-shaped polyhedrins (PH) which are expressed at the very late stage of infection in the nucleus of the infected insect. Unless specifically mentioned, the term recombinant polyhedra means the polyhedra consisting of PH-CP-GFP fusion proteins.

The PH-CP-GFP fusion protein is structured in such a way that the C-terminal of the PH protein is linked to the N-terminal of the CP whose the C-terminal is connected to the N-terminal of the GFP.

As a structural protein, the PH itself is of no

pathogenicity and protects virus particles against the environment. When taken in by insects, PH is solubilized in the alkaline environment of the gut and cleaved by proteases to release virus particles.

5 The CP is a Bt endotoxin, examples of which include Cry1Ac, Cry1Ab, Cry I E, and protein segments containing the pathogenic natures thereof. Preferable is the Cry1Ac protein segment containing the amino acid sequence of No. 1 to No. 613. Like the PH, the CP is solubilized in the
10 alkaline condition of the gut and cleaved to an activated pathogenic segment by proteases. For example, the pathogenic segment of Cry1Ac is the amino acid sequence of No. 27 to No. 607.

Composing the recombinant polyhedra of baculovirus, the
15 fusion protein of the present invention, therefore, is disintegrated in the alkaline environment of the gut and by the action of proteases. That is, the fused PH is decomposed to release viruses while the CP is cleaved to the activated pathogenic segment which, then, binds to the
20 epithelial cells of the gut, disrupting the gut. In result, the insects which take in the fusion protein of the present invention, cease to eat food and show paralytic symptoms owing to the change in the pH of the digestive juice and humor and are finally killed in 24 to 48 hours.

25 The GFP used in the present invention, derived from jellyfish *Aequorea victoria*, is a very stable protein which

emits green light on excitation with ultraviolet. The GFP was introduced in a recombinant virus with the aim of controlling insect pests (Chao et al., *Nature*, 380, 396-397, 1996). In the present invention, the GFP serves to
5 facilitate the detecting and monitoring of the recombinant baculovirus-infected larvae and their distribution in an ecosystem, thereby preventing injudicious abuse of the insecticide.

In the present invention, there was developed a novel
10 recombinant baculovirus, ColorBtrus, which produces a recombinant polyhedra made up of the fusion protein in which an AcNPV-derived PH, a Cry1Ac protein segment of amino acid sequence No. 1 to 613, and a GFP are, in sequence, linked. The baculovirus ColorBtrus was deposited in the Korean
15 Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on March 10, 1998 and received a deposition No. KCTC 0444BP.

A plausible approach to the development of the novel recombinant baculovirus, ColorBtrus, is to construct a
20 baculovirus transfer vector carrying a fusion gene encoding the PH-CP-GFP protein. For the fusion gene, three base sequence fragments which respectively encode PH, CP and GFP, are sequentially linked. Available for the gene encoding PH are those which are derived from AcNPV, *Spodoptera exigua*
25 NPV or *Bombix mori* NPV with preference to the PH gene of

AcNPV. Examples of the CP-coding base sequence include those encoding Cry1Ac (Adang, M.J. et al., Gene, 36, 289-300 91985)), Cry1Ab, Cry1E, or protein segments containing the pathogenic natures thereof. Preferable is the base sequence 5 corresponding to the Cry1Ac protein segment of amino acid sequence No. 1 to 613. As for the GFP-coding base sequence, it preferably contains the GFP gene derived from jellyfish *Aequorea victoria*. These base sequences may be obtained by PCR or enzymatic cleavage from vectors carrying 10 corresponding genes or by chemical synthesis.

These sequences are sequentially linked in such a way that the 5'-end of the PH-coding base sequence and the 3'-end of the GFP -coding base sequence are the opposite ends of the resulting base sequence. This fusion sequence is 15 inserted in a known baculovirus transfer vector, e.g, pAcUW31 (GenBank Registration No. U02452) to give a novel baculovirus transfer vector carrying a fusion gene encoding the PH-CP-GFP fusion protein.

In detail, the GFP gene and the PH gene may be 20 amplified from plasmid pGFP (Clontech) and wild-type AcNPV, respectively, by PCR. The 5'-end of the GFP gene is linked to the 3'-end of the PH gene and the linked sequence is inserted in a baculovirus expression vector PAcUW31 (Clontech) to construct a baculovirus transfer vector 25 pColorPol (Fig. 1b). The Cry1Ac gene is amplified from plasmid pPN6.6 (obtained from Dr. Adang) by PCR and inserted

between the PH gene and the GFP gene on the pColorPol to give a plasmid pColorBtrus (Fig. 1c). In the pColorBtrus, the three genes coding respectively for PH, Cry1Ac and GFP are sequentially fused. Both native polyhedrin and the fusion proteins need to be expressed in the same cell in order to stably produce the recombinant polyhedra. For this, a strategy is to create a recombinant virus with two promoters, p10 and polyhedrin gene promoters which initiate transcription of native polyhedrin and the fusion proteins, respectively, as seen in Figs. 1b and 1c. These two independent transcription units are in opposite directions. In these vector systems, the native PH gene and the fusion genes are introduced downstream the promoter, P_{p10} and P_{PH} , respectively.

15 A DNA solution of the recombinant baculovirus transfer vector thus obtained and the genomic DNA of wild-type baculovirus in 20 mM HEPES buffer is mixed with Lipofectin™ (Gibco) and allowed to stand at room temperature. Thereafter, this Lipofectin-DNA complex solution is dropwise added in a cell culture dish seeded with Sf9 cells (InVitrogen) and incubated to produce recombinant baculoviruses. For this transfection, Sf21 and Tn5 can also be used as host cells. But, preferable is Sf9. An inverted phase contrast microscope is useful to observe the polyhedra. After a predetermined time, the culture is centrifuged and the recombinant virus, ColorBtrus, is

obtained from the supernatant.

The recombinant baculovirus of the present invention shows an insecticidal activity 100 times as large as, and a killing time three times as fast as those of the wild-type 5 AcNPV. For use as an insecticide, the recombinant baculovirus may be formulated into granules, powders, liquid phase agents and aqueous solutions with preference to aqueous solutions. For example, an aqueous solution comprises 2×10^{10} particles of the recombinant baculovirus, 10 100 g of white carbon, 100 g of sucrose, 50 g of polyvinylalcohol, 10 ml of Triton X-100 and 10 g of bentonite, added with water to a final volume of 10 liters.

The formulated insecticide composition of the present invention can be applied to the surfaces of plants, such as 15 stone-leeks, cabbages, mulberry leaves, fruit trees, flowering plants, garden stuffs, etc, as well as to the soils in which the plants grow. In addition, the harvested vegetables can be treated with the insecticide composition during transportation or storage.

20 Within the targets of the insecticide of the present invention, there are included lepidopteran insects, such as *Plutella xylostella*, *Sopdoptera exigua*, and *Hyphantria Cunea*, etc.

A better understanding of the present invention may be 25 obtained in light of the following examples which are set

forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE I : Construction of Baculovirus Transfer Vector

5

(1) Construction of transfer vector pAcG

A GFP gene, derived from jellyfish *Aequorea victoria*, was amplified from pGFP (Clontech) by PCR using the following two primers:

10 [Sequence 1]

5'-AAGGATCCATGAGTAAAGGAGAAGAAC-3'

[Sequence 2]

5'-AACTGCAGCTATTTGTATAGTTCATCCATGCC-3'

The amplified gene product was digested with *Bam*HI and
15 *Pst*I and inserted into the *Bam*HI-*Pst*I site of pBacPAK8 (Clontech) to yield a transfer vector pAcG. In this vector, the GFP gene was located downstream polyhedrin promoter (P_{PH}), as seen in Fig. 1a.

20 (2) Construction of transfer vector pColorPol

A PH gene was amplified from the genomic DNA of wild-type baculovirus AcNPV by PCR using the following two primers:

[Sequence 3]

25 5'-AGTTGCTGATATCATGG-3'

[Sequence 4]

5'-AACTCGAGATACGCCGGACCAGTG-3'

In the Sequence 3, the underlined part represents the recognition site of EcoRV.

5 The amplified gene product was digested with EcoRV and XhoI and inserted into the EcoRV-XhoI site of pBacPAK6 to yield a transfer vector pPol.

Separately, a GFP gene was synthesized from the pGFP by PCR using the following two primers:

10 [Sequence 5]

5'-AACTCGAGATGAGTAAAGGAGAAGAAC-3'

[Sequence 6]

5'-CTATTTGTATAGTTCATCCATGCC-3'

In the Sequence 5, the underlined part represents the recognition site of XhoI.

This GFP gene was digested with XhoI and SnaBI and inserted into the XhoI-SnaBI site of the plasmid pPol to yield a transfer plasmid pPolGFP. After being digested with SnaBI and BamHI, the PH-GFP fusion gene obtained from the pPolGFP was inserted into the SnaBI-BamHI site of an expression vector pAcUW31 (U02452) containing independent double promoters while a wild-type AcNPV PH gene was inserted into the EcoRI-BglIII site of the expression vector to yield a transfer vector pColorPol. In this vector, the GFP-PH fusion gene and the AcNPV PH gene were located downstream the PH promoter (P_{PH}) and the p10 promoter (P_{p10}),

as seen in Fig. 1b.

(3) Construction of transfer vector pColorBtrus

A Cry1Ac DNA fragment was synthesized from a plasmid 5 pPN6.6 containing a Bt. Cry1Ac gene (Adang, M. J. et al., *Gene*, 36, 289-300 (1985)) by PCR using the following two primers:

[Sequence 7]

5'-AACTCGAGATGGATAACAATCCGAAC-3'

10 [Sequence 8]

5'-AACTCGAGTGTTGCAGTAACTGG-3'

In the Sequences 7 and 8, the underlined parts are the recognition site of XhoI.

This Cry1Ac DNA fragment was digested with XhoI, 15 followed by inserting the fragment into the XhoI site of the pColorPol to yield a transfer vector pColorBtrus. Accordingly, a PH-Cry1Ac-GFP fusion gene was located downstream the promoter P_{PH} in the pColorBtrus, as seen in Fig. 1c.

20

EXAMPLE II : Construction of Recombinant AcNPV

1-1.5x10⁶ *Sodoptera frugiperda* Sf cells (InVitrogen, Cat. No. B825-01) seeded in each of cell culture dishes were 25 incubated at 27 °C until they attached onto the dishes.

17

Meanwhile, a DNA solution of 1 μ g of wild-type AcNPV genomic DNA (BacPAK6, Clontech, Cat. No. 6144-1) and 5 μ g of the transfer vector pAcG, pColorPol or pColorBtrus DNA in 20 mM HEPES buffer, whose volume was adjusted with sterile water 5 to 50 μ l of a final volume, was added with an equivolume of Lipofectin™ (Gibco). Subsequently, the mixture was incubated at room temperature for 15 min. The cells were washed twice with 2 ml serum-free TC-100 medium (Sigma). TC-100 (1.5 ml) was added to each dish. The Lipofectin-DNA 10 complexes were added dropwise to the medium covering the cells while gently swirling the dish. Following incubation of the cells at 27 °C for 6 hour, 1.5 ml of TC-100 supplemented with 10% FBS (Gibco BRL) and antibiotics were added to each dish and the incubation at 27 °C continued. 4 15 or 5 days after transfection, the supernatant was taken and centrifuged at 7,000 g for 5 min to obtain recombinant a baculovirus AcG, ColorPol or ColorBtrus. Before centrifugation, the supernatant was preferably observed with an inverted phase contrast microscope (x100) to confirm 20 polyhedra.

EXAMPLE III : Microscopy of Recombinant Polyhedra

The recombinant baculovirus AcG, ColorPol or ColorBtrus 25 was seeded at a population of 2.0×10^6 per 60mmx15mm plate and allowed to stand for 1 hour before infection to Sf9

cells. In regard to infection, about 5 viruses per Sf9 cell were preferred. To characterize the expression of the recombinant polyhedrin or the fusion proteins, the cells infected with the recombinant baculovirus were observed at 4 5 days postinfection by a light microscope and a fluorescent microscope (Axiophot Universal Microscope, Zeiss, x1,000). As a control, a wild-type baculovirus AcNPV (Clontech, Cat. No. K1601-D) was used.

The results are shown in Figs. 2a to 2h. Figs. 2a and 10 2b are respectively a light microphotograph and a fluorescent microphotograph for the Sf9 cells infected with wild-type AcNPV, Figs. 2c and 2d for the Sf9 cells infected with AcG, Figs. 2e and 2f for the Sf9 cells infected with ColorPol, and Figs. 2g and 2h for the Sf9 cells infected 15 with ColorBtrus.

As seen in Figs. 2e to 2h, the fusion proteins of ColorPol and ColorBtrus have normal polyhedra shapes, emitting green light under ultraviolet light. Thus, this microscopy of the cells infected with the recombinant 20 baculovirus ColorPol and ColorBtrus revealed that GFP with fluorescence activity was fused in the polyhedra. In contrast, the polyhedra produced from the wild-type baculovirus AcNPV had a normal appearance, but did not emit green light under ultraviolet light. For AcG, the bright 25 glow of the GFP produced clearly appeared in the whole cells.

EXAMPLE IV : Western Blot Analysis of Recombinant Polyhedra

Sf9 cells were infected with the recombinant baculoviruses AcG, ColorPol and ColorBtrus in the same manner as that of Example III. After incubation at 27 °C, the cells were harvested at 3 days postinfection by centrifugation at 4,000xg for 5 min. After being washed with PBS and mixed with 5x sample buffer [0.6ml 1M Tris-HCl (pH 6.8), 5ml 50% glycerol, 2ml 10% SDS, 0.5 ml β -mercaptoethanol, 1 ml 1% bromophenol blue, 0.9 ml H₂O), the cells were water-boiled at 100 °C for 5 min, allowed to stand at room temperature for a while and then, clarified by centrifugation at 7,000xg for 5 min. The supernatants of the cell lysates were electrophoresed on a 10% polyacrylamide separation gel with a 3% stacking gel containing SDS. The proteins run on the gel were transferred to nitrocellulose membrane. After blotting, the membrane was bound with anti-GFP antibody (Clontech, Cat. No. 8363-2), anti-AcNPV PH antibody (possessed in the Laboratory of Insect Pathology and Genetic Engineering Division of Applied Biology and Chemistry, Seoul National University) Or anti-Bt CryIAc antibody (possessed in the Laboratory of Insect Pathology and Genetic Engineering Division of Applied Biology and Chemistry, Seoul National University) and then, incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at room temperature.

The western blots obtained by use of an anti-GFP antibody, an anti-PH antibody and an anti-Cry1Ac antibody are given as shown in Figs. 3a, 3b and 3c, respectively. As demonstrated from the figures, the polyhedras produced from the recombinant baculoviruses ColorPol and ColorBtrus, were composed of a PH-GFP fusion protein 60 kDa in molecular weight and a PH-Cry1Ac-GFP, respectively.

EXAMPLE V : Electromicroscopy of Immunogold-labeled

10 Recombinant Polyhedra

Samples were prepared by ultracentrifuging the same recombinant baculoviruses ColorBtrus and wild-type AcNPV cultures as in Example III in a gradient sucrose ranging from 40 to 65% and isolating the AcNPV polyhedra and the ColorBtrus polyhedra at the 56% region. These samples were fixed in a 0.1M PBS (pH 7.5) containing 1% (w/v) para-formaldehyde, rinsed with 0.1M glycine (pH 7.5), dehydrated and then, embedded in Lowicryl K4M (polysciences).

20 Ultrathin sections of the embedded polyhedras were treated with an anti-GFP antibody, an anti-Cry1Ac antibody and an anti-PH antibody on nickel grids. These sections were exposed to a secondary antibody, such as colloidal gold-conjugated goat anti-rabbit antibody (Biocell), and stained with 0.2% lead citrate and 2% uranyl acetate before being observed with a transmission electron microscope (Hitachi M-

600).

Referring to Fig. 4, there are transmission electron microphotographs showing the immunogold-labeled recombinant polyhedra. The wild-type AcNPV polyhedra is shown in Fig. 4a and the recombinant ColorBtrus polyhedra in Fig. 4b. In the photographs, GFP, Cry1Ac and PH are discriminated by the gold particles with a diameter of 30 nm, 20 nm and 10 nm, respectively. As seen, gold particles with anti-PH, Cry1Ac and GFP antibodies are concurrently observed in the recombinant polyhedra of ColorBtrus while only gold particles with an anti-PH antibody are shown in Fig. 4a. This transmission electron microscopy, therefore, revealed that the recombinant polyhedra of ColorBtrus was a fusion protein of PH, Cry1Ac and GFP.

15

EXAMPLE VI : Western Blot Analysis of Recombinant Polyhedra Treated with Silkworm Gut Juice

The gut juices vomited from the 5th instar silkworm shocked with an electric current, were added to equivolumes of a wild-type AcNPV polyhedra, the recombinant ColorBtrus polyhedra and a Bt Cry1Ac protein and allowed to stand at room temperature for 5 min. These samples were subjected to 10% SDS-PAGE and Western blot analysis using an anti-Bt Cry1Ac antibody, in the same manner as in Example IV. Upon mock infection, water was used.

The Western blots are shown in Fig. 5. In the ColorBtrus lane, a band of about 65 kDa was detected which is identical to the cleaved, active protein of CryIAc.

Thus, the data demonstrate that the recombinant polyhedra of ColorBtrus taken by insects are immediately solubilized in the alkaline environment of the gut juice and cleaved to the activated toxin by the action of the proteases present therein.

10 EXAMPLE VII : Insecticidal Bioassay of Recombinant Virus

The toxicity of the recombinant virus ColorBtrus was determined against the Diamondback moth, *Plutella xylostella* with a control of wild-type AcNPV. To determine the median lethal dose (LD_{50}), fifty second instar larvae were fed with eight 2-fold series diluted virus stocks (5120, 1280, 640, 320, 160, 80, 40 and 20 polyhedra per larva) on Chinese cabbage leaves. Larvae that consumed the dose in 24 hr were transferred to fresh leaves of Chinese cabbage and examined daily. The mortality was scored for a determined time for the determination of LD_{50} . This procedure was repeated three times.

In order to determine the median survival time ST_{50} , fifty second-instar larvae each were fed with a virus suspension (1500 polyhedra per larva) on Chinese cabbage leaves.

The results are given in Table 1, below.

TABLE 1

Virus	LD ₅₀	ST ₅₀
	(No. polyhedra/larva)	(hr)
wt AcNPV	3,000	153.6
ColorBtrus	29.6	31.8

The improved insecticidal activity of the recombinant polyhedra of ColorBtrus was demonstrated by reduction in both medial lethal dose (LD₅₀) and median survival time (ST₅₀). For *P. Xylostella* larvae, the recombinant virus ColorBtrus shows 100-fold increased insecticidal activity and 5-fold decreased killing time compared with the wild-type AcNPV virus. Therefore, the recombinant virus of the present invention, even though used at a significantly small amount, is able to kill insect pests within a short time.

With reference to Fig. 6, there is shown the accumulated insecticidal activity of the recombinant baculovirus ColorBtrus against *P. Xylostella* larvae. When *P. xylostella* was fed at a dose of 5120 (closed circle ●) and 1280 (open circle ○) recombinant polyhedra per larva, the mortality reached approximately up to 100% and 90%, respectively, in three days. In the latter case, the number of dead larvae started to increase again at the sixth day from the treatment, and the mortality reached 94% finally.

Fig. 7a shows *P. xylostella* larvae which were infected

with the recombinant polyhedra of ColorBtrus three days earlier and Fig. 7b is a fluorescent microphotograph showing the fat body from the dead larvae which was infected with ColorBtrus six days earlier. The larvae which were killed 5 at three days postinfection, exhibited a typical symptom due to Bt Cry1Ac (a) and the production of the recombinant polyhedra by the ColorBtrus was confirmed from the fat body from the dead larvae infected with ColorBtrus at 6 days postinfection (b). These data reveal that the recombinant 10 baculovirus ColorBtrus has a killing power endowed from the crystal protein until 3 days postinfection while killing the larvae by viral replication from 6 days postinfection.

Referring to Fig. 8, there is shown a reduction in feeding damage by ColorBtrus-infected *P. xylostella* larvae. 15 The polyhedra of ColorBtrus (Fig. 8a) and of wild-type AcNPV (Fig. 8b) were treated on Chinese cabbage leaves. As seen, *P. xylostella* larvae treated with the recombinant polyhedra of ColorBtrus caused much less damage to the leaves than did the larvae infected with wild-type AcNPV.

20 According to another aspect of the present invention, foreign proteins can be introduced to the polyhedra of wild type AcNPV, thereby constructing a novel baculovirus expression vector system which expresses many foreign proteins and facilitates their isolation, as apparent in the 25 recombinant baculovirus ColorBtrus. Because almost all of

25

the proteins which are expressed in conventional expression vectors are present in soluble forms, they are difficult to purify and require expensive apparatuses in the purification. In contrast, the novel baculovirus expression system carrying the polyhedra gene expresses the introduced foreign proteins in a form convenient to purify, for example, only by centrifugation. Thus, a significant economical profit can be obtained in terms of time and cost. Examples of the proteins which can be expressed by the baculovirus expression vector system of the present invention, include veterinary medicines such as interferon for cats or dogs, human medicines such as anticancer agents, insulin and interferon, antibacterial agents, antibiotics, etc.

In addition, the recombinant polyhedra of the present invention can be applied for vaccine development. If a gene encoding foreign protein for use as a vaccine is fused to the gene encoding the polyhedrin protein in the baculovirus expression vector system, the foreign protein can be produced in a crystalline form along with the polyhedrin protein which is harmless to the body, so that the protein can maintain its vaccinal function without the aid of an adjuvant.

25 **INDUSTRIAL APPLICABILITY**

As described hereinbefore, the recombinant baculovirus ColorBtrus of the present invention expresses polyhedra made up of a PH-CP-GFP fusion protein the CP moiety of which is solubilized in the alkaline environment of an insect's gut juice and cleaved to an active form by proteases while the virions are released. Thus, a baculovirus insecticide of the recombinant polyhedra of ColorBtrus exerts the double action mode of Bt crystal protein and viral replication of AcNPV on insects, improving its pathogenicity and host range. Moreover, because the recombinant polyhedra emits green fluorescence under UV light, it can be applied for monitoring and/or detecting infected insects in fields. In addition, this watching device can prevent the insecticide from being overused.

The present invention has been described in an illustrative manner, and it is to be understood the terminology used is intended to be in the nature of description rather than of limitation.

Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

CLAIMS

1. A construction method of a recombinant baculovirus which produces a recombinant polyhedra containing foreign 5 proteins.

2. A recombinant baculovirus, which produces a recombinant polyhedra made up of a baculovirus polyhedrin, a *Bacillus thuringiensis* crystal protein and jellyfish 10 *Aequorea victoria* green fluorescent protein.

3. A recombinant baculovirus as set forth in claim 2, wherein said baculovirus polyhedrin is derived from *Autographa californica* Nucleopolyhedrovirus and said 15 *Bacillus thuringiensis* crystal protein is a Cry1Ac protein segment containing the amino acid sequence of No. 1 to No. 613.

4. A recombinant baculovirus as set forth in claim 2, 20 having a Deposition No. KCTC 0444BP.

5. A recombinant baculovirus as set forth in claim 2, wherein said recombinant polyhedra is a fusion protein in which said baculovirus polyhedrin, said *Bacillus* 25 *thuringiensis* crystal protein and said jellyfish *Aequorea*

victoria green fluorescent protein are sequentially linked from N-terminal to C-terminal.

6. A transfer vector, carrying a gene encoding a fusion protein in which a baculovirus polyhedrin, a *Bacillus thuringiensis* crystal protein and a jellyfish *Aequorea victoria* green fluorescent protein are directly linked from N-terminal to C-terminal, in sequence.

10 7. A transfer vector as set forth in claim 6, which is plasmid pColorBtrus.

8. A construction method of baculovirus transfer vector pColorBtrus, comprising the steps of:

15 synthesizing a green fluorescent protein-coding DNA fragment from plasmid pGFP by a polymerase chain reaction; synthesizing a polyhedrin gene from wild-type *Autographa californica* Nucleopolyhedrovirus by a polymerase chain reaction;

20 inserting the green fluorescent protein-coding DNA fragment and the polyhedrin gene in baculovirus expression vector pAcUW31 in such a way that the 5'-end of the green fluorescent protein-coding DNA fragment is linked to the 3'-end of the polyhedrin gene, to yield baculovirus transfer
25 vector pColorPol;

29

synthesizing a Cry1Ac gene from plasmid pPN6.6 carrying a *Bacillus thuringiensis* Cry1Ac gene by a polymerase chain reaction; and

inserting the Cry1Ac gene between the polyhedrin gene 5 and the green fluorescent protein-coding DNA fragment.

9. A method of constructing the recombinant baculovirus of claim 2, comprising the steps of:

introducing a transfer vector carrying a fusion gene 10 encoding a fusion protein in which a baculovirus polyhedrin, a *Bacillus thuringiensis* crystal protein and a jellyfish *Aequorea victoria* green fluorescent protein are directly linked from N-terminal to C-terminal, in sequence, and a wild-type baculovirus into an insect cell, simultaneously; 15 and

culturing the cell.

10. A method as set forth in claim 9, wherein said transfer vector is pColorBtrus.

20

11. An insecticidal composition, comprising the recombinant virus of claim 2.

12. Use of the recombinant polyhedra of claim 5 as a 25 vaccine.

13. A purification method of proteins using the transfer vector system of claim 6.

FIG. 1a



FIG. 1b

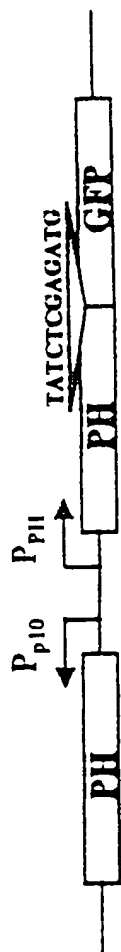


FIG. 1c



2/8

FIG. 2a

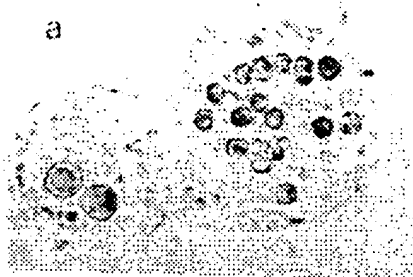


FIG. 2b



FIG. 2c

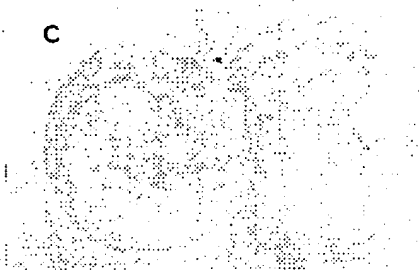


FIG. 2d



3/8

FIG. 2e

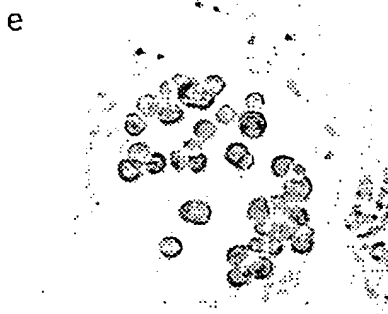


FIG. 2f



FIG. 2g

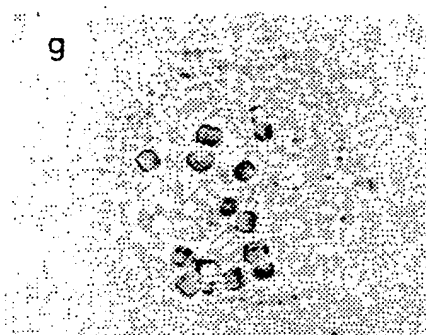
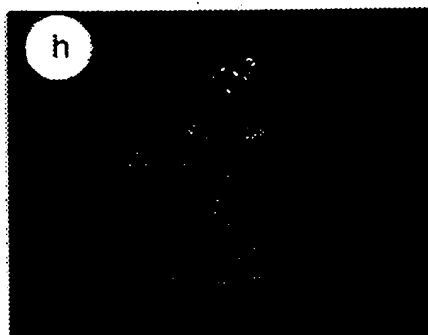


FIG. 2h



4/8

FIG. 3a

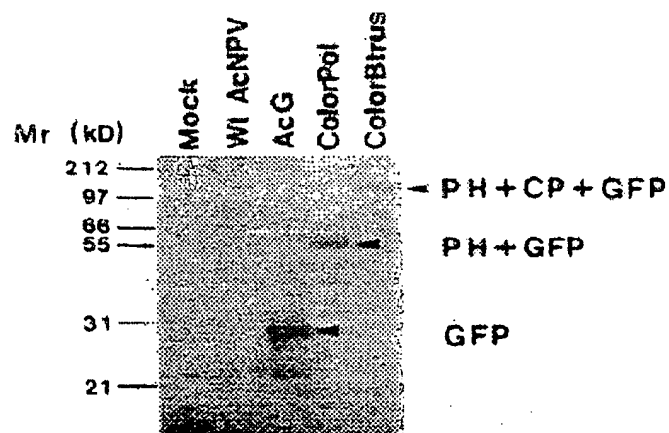


FIG. 3b

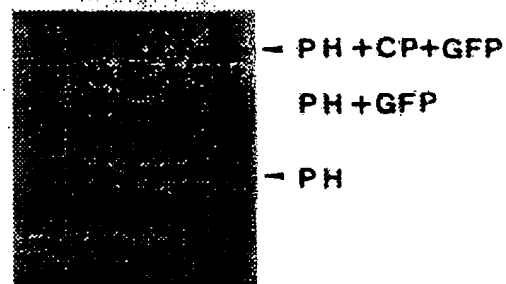


FIG. 3c

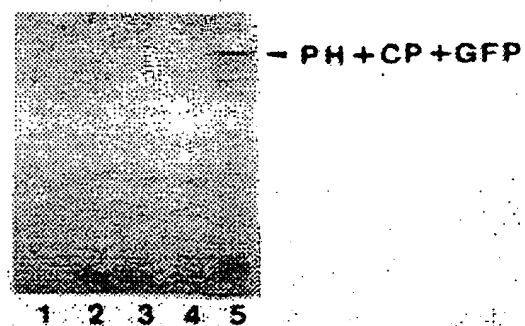


FIG. 4a

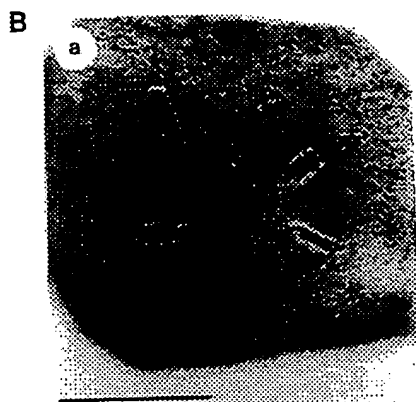
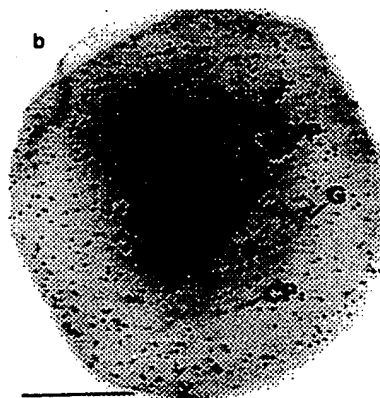
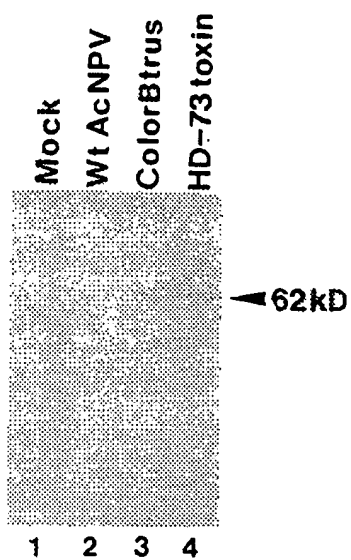
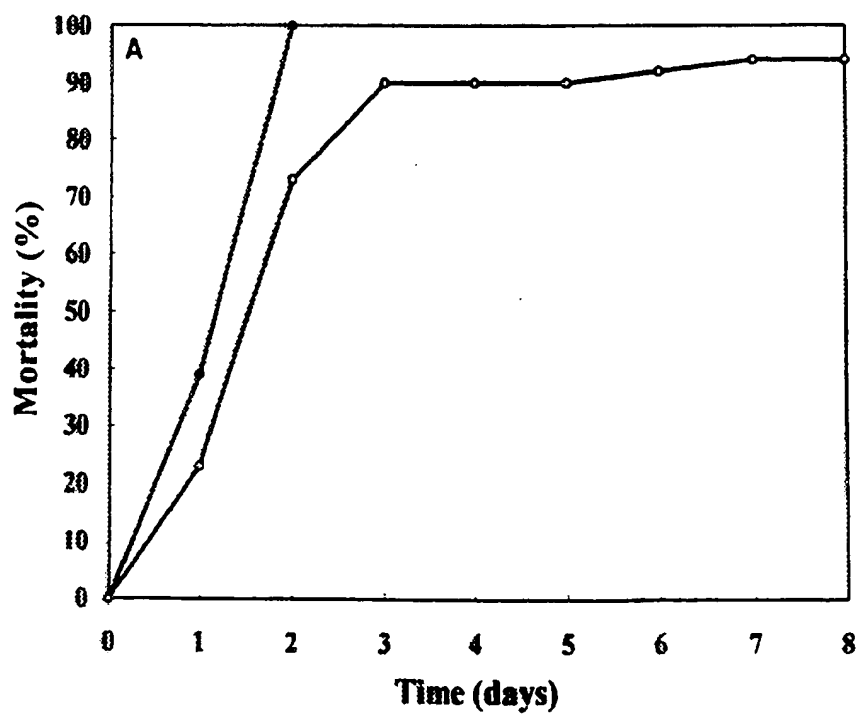


FIG. 4b



6/8

FIG. 5**FIG. 6**

7/8

FIG. 7a



FIG. 7b

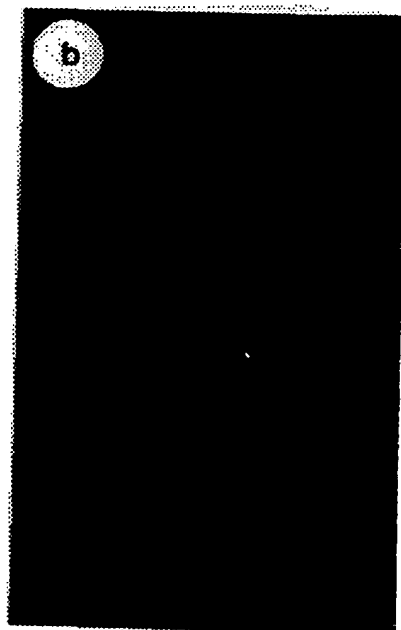
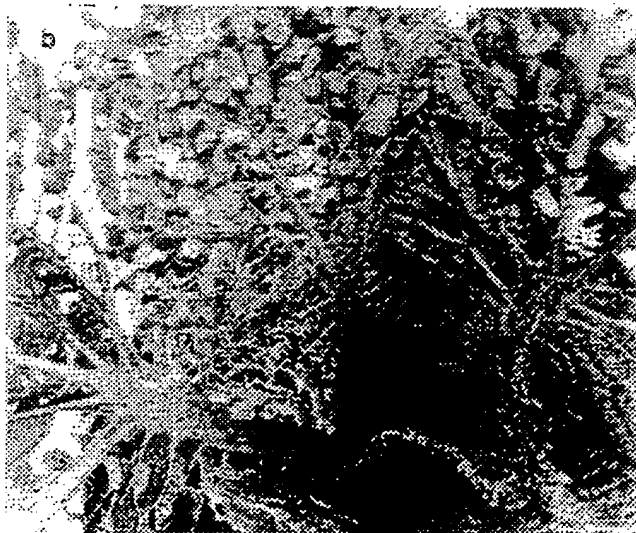


FIG. 8a



FIG. 8b



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 98/00338

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 N 7/01, 15/33, 15/32, 15/62; A 61 K 39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 N 7/01, 15/33, 15/32, 15/62; A 61 K 39/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/26 410 A2 (ZENECA LIMITED) 05 October 1995 (05.10.95), abstract.	1
X	WO 88/07 082 A1 (AMERICAN BIOGENETIC SCIENCES) 22 September 1988 (22.09.88), abstract.	1
X	GB 2 228 486 A (UNIVERSITY OF OTTAWA) 29 August 1990 (29.08.90), abstract.	1

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

„A“ document defining the general state of the art which is not considered to be of particular relevance

„E“ earlier application or patent but published on or after the international filing date

„L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

„O“ document referring to an oral disclosure, use, exhibition or other means

„P“ document published prior to the international filing date but later than the priority date claimed

„T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

„X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&“ document member of the same patent family

Date of the actual completion of the international search

04 May 1999 (04.05.99)

Date of mailing of the international search report

31 May 1999 (31.05.99)

Name and mailing address of the ISA/AT

Austrian Patent Office

Kohlmarkt 8-10; A-1014 Vienna

Facsimile No. 1/53424/200

Authorized officer

Wolf

Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 98/00338

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication	
WO A2	9526410	05-10-1995	AU A1	19570/95	17-10-1995
			AU B2	688011	05-03-1998
			BR A	9507195	09-09-1997
			CA AA	2185234	05-10-1995
			CN A	1147275	09-04-1997
			EP A1	752001	08-01-1997
			GB A0	9405951	11-05-1994
			JP T2	9510871	04-11-1997
			NZ A	282463	27-05-1998
			US A	5885569	23-03-1999
			WO A3	9526410	02-11-1995
WO A1	8807082	22-09-1988	US A	4870023	26-09-1989
			AU A1	17176/88	10-10-1988
			EP A1	349594	10-01-1990
			EP A4	349594	26-09-1990
			JP T2	2502876	13-09-1990
			AU A1	15424/88	10-10-1988
			CA A1	1325610	28-12-1993
			CA A1	1325611	28-12-1993
			EP A1	349583	10-01-1990
			EP A4	349583	05-09-1990
			JP T2	2502873	13-09-1990
			WO A1	8807087	22-09-1988
			US A	5041379	20-08-1991
GB A	2228486		GB A0	8904426	12-04-1989
			GB A1	2228486	29-08-1990

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.